

(19)



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0 191 748
A1

(12)

EUROPEAN PATENT APPLICATION

(21) Application number: 86870013.9

(51) Int. Cl.: C 12 N 15/00, A 61 K 39/015

(22) Date of filing: 03.02.86

(30) Priority: 07.02.85 US 598115

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(43) Date of publication of application: 20.08.86
Bulletin 86/34

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(84) Designated Contracting States: AT BE CH DE FR GB IT
LI LU NL SE

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(54) Malaria vaccine.

(57) A vaccine for protecting mammals against malaria is prepared by a process comprising inserting a coding sequence for all or a portion of the repeat unit of the CS protein of *Plasmodium falciparum* into an *E. coli* expression vector such that the coding sequence is operatively linked to a regulatory element, culturing the transformed *E. coli* such that the polypeptide is produced and purifying the polypeptide therefrom.

EP 0 191 748 A1

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- 1 -

10 BACKGROUND OF THE INVENTION

 Malaria is a severe, widespread disease for
which, despite years of extensive efforts, a vaccine has
not been developed. See, for example, Science, Volume
226, page 679 (November 9, 1984). Experimentally,
15 mammals, including man, have been protected against
infection by the etiologic agent of malaria, Plasmodium,
by vaccination with irradiated sporozoites. Clyde et al.,
Am. J. Trop. Med. Hyg., Volume 24, page 397 (1975) and
Rieckman et al., Bull. WHO, Volume 57 (Supp. 1), page 261
20 (1979). Yoshida et al., Science, Volume 207, page 71
(1980) report that such protection is at least partially
mediated by antibody directed against a protein on the
surface of the sporozoite, the circumsporozoite (CS)
protein; monoclonal antibodies raised against CS proteins
25 neutralize infectivity in vitro and protect animals in
vivo. The CS protein appears to be highly evolutionarily
conserved within species, but is quite varied across
species.

 Four species of Plasmodium are known to infect
30 man. These are P. falciparum, P. vivax, P. ovale and P.
malariae, the latter two occurring at much lower
frequency. Other species of scientific interest are P.
berghei and P. knowlesi, the hosts of these species being,
respectively, rodents and monkeys.

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1 The CS prot in of P. knowlesi comprises twelve
tandem repeats of a twelve amino acid sequence. Zavala et
al., J. Exp. Med., Volume 157, page 1947 (1983), report
that the repeat unit is the major immunogen on the P.
5 knowlesi CS protein, based on experiments showing that
monoclonal antibodies to the repeat unit blocked access of
anti-sporozoite antisera to solubilized sporozoite
protein. Gysin et al, J. Exp. Med., Volume 160, page 935
(1984), reported that a synthetic 24 residue peptide
10 representing tandem repeat units of the P. knowlesi CS
protein neutralized infectivity of virulent sporozoites in
monkeys.

Colman et al., WO 84-2922-A, published August 2,
1984, report cloning of a portion of the coding region for
15 the P. knowlesi CS protein repeat unit and expression of
beta-lactamase and beta-galactosidase fusions thereof in
E. coli. Nussenzweig et al., U.S. 4,466,917, disclose a
sporozoite protein referred to as the P44 protein and its
cloning and expression in E. coli.

20 Enea et al., Proc. Natl. Acad. Sci. USA, Volume
81, page 7520 (1984), report an analogous repeat unit
structure within the CS protein of P. cynomologi.

Kemp et al., WO.84-02917-A, disclose cloning and
expression of P. falciparum cDNA in E. coli.

25 Dame et al., Science, Volume 225, page 593
(1984), report cloning and expression of the CS protein of
P. falciparum in E. coli. The protein is described as
comprising about 412 amino acids with an approximate
molecular weight of 44,000. It comprises 41 tandem
30 repeats of a tetrapeptide. Synthetic 7-, 11- and 15-
residue peptides derived from the repeat region bound to
monoclonal antibodies raised against the CS protein.

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SUMMARY OF THE INVENTION

One aspect of the invention is an E. coli expression vector having a coding sequence for all or a portion of the repeat unit of the CS protein of Plasmodium
5 falciparum.

In other aspects, the invention comprises E. coli transformed with the expression vector of the invention and a process for purifying a polypeptide having four or more tandem repeat units of the Plasmodium falciparum CS
10 protein of the invention from a producing E. coli culture.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1a is a partial restriction endonuclease cleavage map of a region of P. falciparum genomic DNA
15 which carries the coding sequence for the CS protein.

Figure 1b is a partial restriction endonuclease cleavage map of pAS1.

DETAILED DESCRIPTION OF THE INVENTION

20 The polypeptide of this invention comprises four or more tandem CS protein repeat units produced in E. coli. It is not the CS protein, although it may comprise portions of the CS protein other than the repeat unit. The P. falciparum repeat unit is a tetrapeptide having the
25 following sequence:

asparagine(asn)-alanine(ala)-asn-proline(pro)-.

Within the polypeptide of the invention,, variation of the
30 tetrapeptide may be present, provided such does not significantly, adversely affect the reactivity of antibodies thereto with the P. falciparum CS protein. For example, as disclosed by Dame et al., Science, Volume 225, page 593 (1984), which is herein incorporated by reference
35 as though fully set forth, of the 41 tetrapeptide repeats in the naturally occurring P. falciparum CS protein, 37

0191748
1 are asn-ala-asn-pro and 4 are asn-valine (val)
aspartic acid(asp)-pro. Preferably, more than half of the
tetrapeptide repeat units in the polypeptide of the
invention are the so-called consensus sequence,
5 asn-ala-asn-pro.

Preferably, the polypeptide of the invention
comprises about 8 repeats, that is 32 amino acids, up to
about 148 repeats. More preferably, the polypeptide
comprises from about 16 to about 112 repeats.

10 The polypeptide of the invention can be a hybrid,
that is, a fusion polypeptide, having non-CS protein
repeat unit sequences. Such non-CS protein repeat
sequence can serve as a carrier molecule to enhance
immunogenicity or to facilitate cloning and expression in
15 recombinant microorganisms. Alternatively, such
additional sequence can carry one or more epitopes for
other sporozoite immunogens, other Plasmodium immunogens
and/or other non-Plasmodium immunogens. Specifically
excluded from the invention is the CS protein which has
20 been found not to be stably expressed in practicable
amounts in E. coli and not to be necessary for
immunization against P. falciparum.

Specific embodiments of types of polypeptides of
the invention exemplified herein are:

25 Rtet₃₂ polypeptides, which comprise at least 4
repeats with about 32 N-terminal amino acids from the
tetracycline resistance (tetR) gene in pBR322 fused to the
C-terminus of the repeats;

30 Rtet₈₆ polypeptides, which comprise at least 4
repeats with a tetR gene product fused to the C-terminus
of the repeats;

RNS1 polypeptides which comprise at least 4
repeats with the 227 amino acids of NS1 fused to the
C-terminus of the repeats;

35 NS1R polypeptides, which comprise at least 4
repeats with 81 N-terminal amino acids of NS1 fused to the
N-terminus of the repeats;

1 RG polypeptides which comprise at least 4 repeats
followed by a -glycine residue at the C-terminus of the
repeats;

5 RLA polypeptides which comprise at least 4
repeats followed by -leucine-arginine residues at the
C-terminus of the repeats; and

RN polypeptides, which comprise at least 4
repeats followed by -asn-thr-val-ser-ser at the C-terminus
of the repeats.

10 A genetic coding sequence for the CS protein
repeat units can be obtained by known techniques. These
include synthesis and, preferably, by obtainment from P.
falciparum by reverse transcription of messenger RNA as
disclosed, for example, by Ellis et al., Nature, Volume
15 302, page 536 (1983), or by directly cloning the intact
gene from P. falciparum genomic DNA as disclosed, for
example, by Dame et al., cited previously. The Figure
illustrates the CS protein coding region. P. falciparum,
and sporozoites thereof, can be obtained from infected
20 humans and mosquitoes.

Having cloned the coding sequence for all or part
of the CS protein, a sub-fragment thereof coding for all
or a portion of the repeat unit can be prepared by known
techniques. Figure 1a shows selected available
25 restriction sites within the CS protein gene. Preferred
sites are the Xho II sites. Cutting with Xho II releases
a coding sequence for 16 repeats as follows:

30 N-asn-pro[(asn-ala-asn-pro)₁₅(asn-val-asn-pro)₁]_nC.

wherein n is one. Use of multiple tandem Xho II fragments
in proper orientation results in longer repeats, that is,
n is greater than one.

35 Techniques for synthesizing are well-known and
can be accomplished using commercially available DNA
synthesizers. A synthetic oligonucleotide, having codons

1 for substantially the same amino acids and having the same
Xho II ends or different cleavage sites at the ends, can
be synthesized. Such synthetic oligonucleotides may vary
from the natural 64 codons and may code for the same amino
5 acids or for a polypeptide having a small number,
preferably less than about 8, different amino acids,
provided these do not significantly adversely affect the
immunoprotectiveness of the polypeptide. An exemplary
synthetic coding sequence codes entirely for the consensus
10 sequence, (asn-ala-asn-pro)_n, wherein n is at least 4.

The coding sequence for the polypeptide can be
inserted into any E. coli expression vector, many of which
are known and available. The high level of expression of
the polypeptides of the invention in E. coli is surprising
15 in view of the unusual amino acid composition of the
products - about 50% asparagine (asn), 25% alanine (ala)
and 25% proline (pro). As described further below, it has
been found that the coding sequence is expressed well
using a regulatory element comprising the PL promoter of
20 lambda and the cII ribosome binding site of lambda, as
comprised by the plasmid pAS1, described by Rosenberg et
al., Meth. Enzym., Volume 101, page 123 (1983) and
Shatzman et al., in Experimental Manipulation of Gene
Expression, edit. by M. Inouye, Academic Press, New York,
25 1982. pAS1 carries the pBR322 origin of replication, an
ampicillin resistance marker and a series of fragments
from lambda, including PL, N antitermination function
recognition sites (NutL and NutR), the rho-dependent
transcription termination signal (tRL) and the cII
30 ribosome binding site, including the cII translation
initiation site, the G residue of which is followed
immediately by a Bam HI cleavage site. pAS1 can be
derived from pKC30cII by deleting nucleotides between the
Bam HI site at the cII-pBR322 junction of pKC30cII and the
35 cII ATG and religating the molecule to regenerate the Bam
HI site immediately downstream of the ATG. pKC30cII is

C191748

1 constructed by inserting a 1.3 kb Hae III fragment from
lambda which carries the cII gene into the Hpa I site of
pKC30. See Shatzman et al., cited above, and Rosenberg et
al., cited above. pKC30 is described by Shimitake et al.,
5 Nature, Volume 292, page 128 (1981). It is a pBR322
derivative having a 2.4 kb Hind III-Bam HI fragment of
lambda inserted between the Hind III and Bam HI sites in
the tetR gene of pBR322. A construction similar to pAS1
is described by Courtney et al., Nature, Volume 313, page
10 149 (1985). pAS1 was deposited in the American Type
Culture Collection, Rockville, Maryland, under accession
number ATCC in accordance with the terms of the
Budapest Treaty. The coding sequence is operatively
linked, that is, in correct orientation and in proper
15 reading frame, to a regulatory element of an E. coli
expression vector by standard techniques to construct an
expression vector of the invention.

The polypeptide so expressed is isolated and
purified from the producing culture by standard protein
20 isolation techniques, many of which are well known in the
art. An exemplary, useful purification scheme comprises
1) disruption of cells, 2) clarification of cellular
debris, 3) separation of the polypeptides of the invention
from other polypeptides present in the clarified cell
25 extract and 4) final purification to remove residual
contaminants including residual polypeptides,
carbohydrates, nucleic acids and/or lipopolysaccharides.

The first step can be accomplished such as by
addition of lysozyme or other lysing or permeabilizing
30 agent or by mechanical or ultrasonic disruption. Prior to
centrifugation or filtration to clarify the extract, a
surfactant is added to keep the polypeptide of the
invention in solution.

As one aspect of the present invention, it has
35 been discovered that certain of the polypeptides of the
invention can very efficiently be separated from other

C191748

1 polypeptides by heating the clarified extract to about
80°C following addition of a detergent to maintain
solubility of the protein. Heating to 80°C for at least
about 4 minutes was discovered to cause nearly all
5 bacterial polypeptides to precipitate without denaturing
polypeptides comprised substantially of the repeats or of
the repeats fused to other non-heat-denaturable
sequences. The denatured bacterial polypeptides can be
pelleted by centrifugation and removed. This procedure
10 has been used to purify Rtet₃₂, RG, RLA and Rtet₈₆
polypeptides. In particular, this procedure was used to
purify successfully R16tet₃₂, R32tet₃₂, R48tet₃₂,
R64tet₃₂, R48G, R32LA and R16tet₈₆, as described in
the Examples, below, but heating of R16NS1 and R32NS1
15 resulted in precipitation of these polypeptides.

The polypeptide of the invention can be further
purified such as by addition of a selective precipitating
agent, followed by a final chromatographic step such as
ion exchange chromatography or reverse phase HPLC.

20 In the vaccine of the invention, an aqueous
solution of the polypeptide of the invention, preferably
buffered at physiological pH, can be used directly.
Alternatively, the polypeptide, with or without prior
lyophilization, can be admixed or adsorbed with any of the
25 various known adjuvants. Such adjuvants include, among
others, aluminum hydroxide, muramyl dipeptide and saponins
such as Quil A. As a further exemplary alternative, the
polypeptide can be encapsulated within microparticles such
as liposomes. In yet another exemplary alternative, the
30 polypeptide can be conjugated to an immunostimulating
macromolecule, such as killed Bordetella or a tetanus
toxoid.

Vaccine preparation is generally described in New
Trends and Developments in Vaccines, edited by Voller et
35 al., University Park Press, Baltimore, Maryland, U.S.A.,
1978. Encapsulation within liposomes is described, for

1 example, by Fullerton, U.S. Patent 4,235,877. Conjugation
of proteins to macromolecules is disclosed, for example,
by Likhite, U.S. Patent 4,372,945 and by Armor et al.,
U.S. Patent 4,474,757. Use of Quil A is disclosed, for
5 example, by Dalsgaard et al., Acta. Vet. Scand., Volume
18, page 349 (1977).

The amount of polypeptide present in each vaccine
dose is selected as an amount which induces an
immunoprotective response without significant, adverse
10 side effects in typical vaccinees. Such amount will vary
depending upon which specific polypeptide is employed and
whether or not the vaccine is adjuvanted. Generally, it
is expected that each dose will comprise 1 - 1000 ug of
polypeptide, preferably 10 - 200 ug. An optimal amount
15 for a particular vaccine can be ascertained by standard
studies involving observation of antibody titres and other
responses in subjects. Following an initial vaccination,
subjects will preferably receive a boost in about 4 weeks,
followed by repeated boosts every six months for as long
20 as a risk of infection exists.

The following Examples are illustrative, and not
limiting, of the invention. The CS protein coding
sequence was supplied by James Weber, Walter Reed Army
Institute for Research, as a 2337 bp Eco RI fragment (see,
25 Fig. 1a) of λ MPF1 (Dame et al., cited above) in the Eco RI
site of pUC8, a standard E. coli cloning vector
(available, for example, from Bethesda Research
Laboratories, Inc., Gaithersburg, MD). The resulting pUC8
derivative is referred to as pUC8 clone 1.
30

EXAMPLES

Example 1. CS Protein Derivative

Purified pUC8 clone 1 plasmid DNA (40 ug) was
35 digested with restriction endonucleases StuI and RsaI (100
units of each enzyme) in 400 ul of medium buffer (50 mM

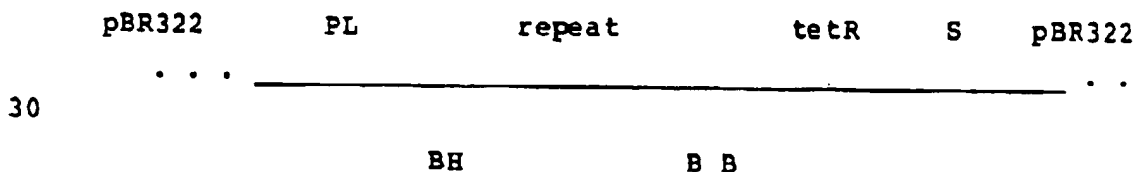
C191748

1 Tris, pH7.5, 50mM NaCl, 1mM dithiothreitol (DTT), 10mM
MgCl₂) for 1.5 hours at 37°C. The resulting 1216 base
pair fragment, encoding all but the first 18 amino acids
of the CS protein was isolated by electrophoresis on a 5%
5 polyacrylamide gel (PAGE). Expression vector pAS1 (10 ug)
was digested with restriction endonuclease Bam HI (25
units) in 200 ul medium buffer for 1.5 hours at 37°C. The
cut plasmid was then treated with DNA polymerase large
fragment (Klenow, 5 units; 20 mM Tris-HCl, pH7.5, 7mM
10 MgCl₂, 60mM NaCl, 6mM 2-mercaptoethanol and 0.25mM of
each of the four deoxynucleotide triphosphates; 25°C, 15
minutes) to end fill the Bam HI site. The CS gene
fragment (1 ug) was then ligated into this vector (100ng)
in 30 ul ligase buffer (50mM Tris, pH7.5, 1mM DTT, 10mM
15 MgCl₂, 100 uM rATP) with one unit of T4-DNA ligase for
16 hours at 4°C. The ligation mixture was transformed
into E. coli strain MM294CI+, and ampicillin resistant
colonies were obtained, and screened for insertion of the
CS gene fragment into the pAS1. A plasmid with the
20 correct construction (pCSP) was identified and was
transformed into E. coli strain N5151 (cIts857) and tested
for expression of the full length CS protein. (The 18
amino acid deletion at the amino terminus of the protein
would correspond to a cleaved signal peptide of the
25 authentic CS protein.) Cells were grown in Luria-Bertani
Broth (LB) at 32°C to an absorbance at 650nm (A₆₅₀) of
0.6 and temperature induced at 42°C for 2 hours to turn on
transcription of the PL promoter of the expression plasmid
and subsequent translation of the CS protein derivative.
30 Cells were sampled in 1 ml aliquots, pelleted, resuspended
in lysis buffer (10mM Tris-HCl, pH7.8, 25% (vol/vol)
glycerol, 2% 2-mercaptoethanol, 2% sodium dodecyl sulfate
(SDS), 0.1% bromophenyl blue) and incubated in a 105°C
heating block for 5 minutes. Proteins were separated by
35 SDS-PAGE (13% acrylamide, 30:0.8 acrylamide:
bis-acrylamide ratio). Proteins were transferred to

0191748
1 nitrocellulose and the CS protein produced in E. coli was
detected by western blot analysis using a pool of five
monoclonal antibodies reactive with the tetrapeptide
repeat domain of the P. falciparum CS protein. (Dame et
5 al., cited previously.)

Example 2. R16tet₈₆

Purified pUC8 clone 1 plasmid DNA (100 ug) was
digested with restriction endonuclease Xho II (40 units)
10 in 400 ul medium buffer at 37°C for 16 hours. A 192 base
pair fragment encoding 16 tetrapeptide repeats of the CS
protein was then isolated by PAGE. Expression vector pAS1
was cleaved with restriction endonuclease Bam HI as
described in Example 1. The 192 base pair Xho II fragment
15 (1 ug) was ligated into the Bam HI site of pAS1 (100ng) as
described in Example 1. The ligation mix was transformed
into E. coli strain MM294CI+. A clone was identified
which contained a single 192 base pair Xho II fragment in
the correct orientation at the Bam HI site of pAS1 by
20 polyacrylamide gel electrophoresis analysis of a Bam
HI-Hind II fragment of the plasmid, the Hind II site being
downstream of the tetR gene and the Bam HI site being at
the juncture of the cII ATG and the insert in correctly
oriented plasmids. This plasmid pR16tet₈₆ is
25 illustrated as follows:



wherein BH represents a Bam HI site, B represents a Ban II
site and S, a termination codon. The pR16tet₈₆ was used to
35 transform E. coli strain N5151 (cIts857) and examined for
production of the CS protein tetrapeptide repeat by western

0191748
1 blot analysis. The protein so produced had the following sequence:

N-met-asp-pro(asn-ala-asn-pro)₁₅(asn-val-asp-pro)₁ T86-C

5 wherein T86 was 86 amino acids derived from the tetracycline resistance gene present on pAS1. The N-terminal methionine (met) residue was also derived from the vector, more particularly, from the cII protein
10 initiation codon.

Example 2A. R32tet₈₆ and R48tet₈₆

Purified pR16tet₈₆ plasmid DNA (10 ug) was digested with 25 units of Bam HI in 200 ul of medium
15 buffer for 2 hours at 37°C. One hundred ng of this DNA was then ligated with 1 ug of the 192 base pair Xho II fragment as described above. Plasmid expression vectors, pR32tet₈₆ and pR48tet₈₆, coding for the following polypeptides were prepared and expressed in E. coli.

20 N-met-asp-pro[(asn-ala-asn-pro)₁₅-(asn-val-asp-pro)₁]_n-T86-C

wherein n is 2 (R32tet₈₆) or n is 3 (R48tet₈₆). pAS1 clones wherein n was 2 or 3 were selected from clones in
25 which n was other than 2 or 3, respectively, as described above. All clones examined had the insert in the correct orientation. Both R32tet₈₆ and R48tet₈₆ were expressed at approximately the same levels as R16tet₈₆, as estimated by immunoblotting.

30 Immunoblot analysis of several of the Rtet₈₆ proteins revealed a heterogeneous set of products which could not be seen by Coomassie Brilliant Blue R-250 staining. These proteins appeared to have accumulated to roughly half the amounts of the Rtet₃₂ polypeptides,
35 described below. It appeared that the sizes of the smallest degradation products were proportional to the

0191748

1 number of tetrapeptide repeats in the clones. The
instability of these proteins may be due to degradation of
the heterologous COOH-terminal tail.

5 Example 3. R16tet₃₂

Purified pR16tet₈₆ DNA (10 ug) was cut with 25
units of restriction endonuclease Ban II in 200 ul of
medium buffer for 2 hours at 37°C. One hundred nanograms
of the cut DNA was then ligated closed. This manipulation
10 resulted in the deletion of a 14 base pair Ban II fragment
and produced a termination codon just downstream of the
remaining Ban II site. The resulting plasmid,
pR16tet₃₂, was used to express R16tet₃₂ in E. coli
strain N5151 and R16tet₃₂ was purified therefrom.

15 Thirty grams (wet weight) of E. coli containing R16tet₃₂
were resuspended in 200 ml buffer A (50mM Tris HCl, pH
8.0, 2mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM
dithiothreitol, 5% (vol/vol) glycerol). Lysozyme was
added to a final concentration of 0.2 mg/ml, and the
20 mixture was incubated on ice for 30 minutes to lyse
cells. The mixture was then treated in a Waring blender
for 3 minutes at the high setting followed by sonication
for one minute with a Branson 350 sonifier to shear
bacterial DNA. Sodium deoxycholate was added to a final
25 concentration of 0.1% (w/v), and this mixture was stirred
for 30 minutes at 4°C. The suspension was then
centrifuged at 12,000 x g for 30 minutes to remove cell
debris. The supernatant was collected in a flask,
incubated in a boiling water bath for 10 minutes, and
30 centrifuged at 12,000 x g for 30 minutes. It was found
that nearly all E. coli proteins precipitated during the
heat step and pelleted during the centrifugation, whereas,
the R16tet₃₂ protein was soluble and was contained in
the supernatant. The supernatant was collected and
35 ammonium sulfate was then slowly added to a final
concentration of 20% of saturation. This resulted in

C191748

1 selective precipitation of the R16t₃₂ protein which
was then collected by centrifugation (12,000 x g for 30
minutes). At this point the R16tet₃₂ protein was
greater than about 95% pure with respect to other
5 contaminating bacterial proteins.

A final chromatographic step (e.g., ion exchange,
reverse phase high performance liquid chromatography,
phenyl sepharose chromatography, size separation, etc.)
can then be performed to remove residual contamination by
10 other materials such as proteins, carbohydrates, nucleic
acids or lipopolysaccharides. R16tet₃₂ was expressed
and purified at levels approximately equal to 5% of total
E. coli protein, that is, about 30-60 mg/L, as shown by
Coomassie Blue Staining.

15 R16tet₃₂ has the following sequence:

N-met-asp-pro[(asn-ala-asn-pro)₁₅(asn-val-asp-pro)₁]_nT32-C

wherein n is one and T32 is 32 amino acids derived from
20 the tetracycline resistance gene. More particularly, T32
has the following sequence:

-leu-arg-arg-thr-his-arg-gly-arg-his-his-arg-arg-his-arg-cys
-gly-cys-trp-arg-leu-tyr-arg-arg-his-his-arg-trp-gly-arg-ser
25 -gly-ser-C

the remaining Ban II site being between residues 30 and 31.

Example 3A. R32tet₃₂, R48tet₃₂

30 Substantially as described in Example 3, above,
R32tet₃₂ and R48tet₃₂, (R16tet₃₂ in which n is 2 and
3, respectively), were expressed in E. coli and isolated to
the same level and degree of purity as R16tet₃₂. The
starting vectors were pR32tet₈₆ and pR48tet₈₆,
35 respectively.

C191748

1 Example 3B. R64tet₃₂, R80tet₃₂

Purified pR48tet₃₂ plasmid DNA (10 ug) was digested with 25 units of Bam HI in 200 ul of medium buffer for 2 hours at 37°C. One hundred nanograms of this DNA was then ligated with 1 ug of the 192 base pair Xho II fragment as described above. Plasmid expression vectors coding for the following polypeptides were prepared and expressed in E. coli.

10 N-met-asp-pro[(asn-ala-asn-pro)₁₅(asn-val-asp-pro)₁]_n-T32-C

wherein n is 4 (R64tet₃₂) or n is 5 (R80tet₃₂). pAS1 clones wherein n was 4 or 5 were selected from clones in which n was other than 4 or 5, respectively, as described above. Both R64tet₃₂ and R80tet₃₂ expressed at approximately the same levels as R48tet₃₂. R64tet₃₂ was purified in substantially the same manner as R16tet₃₂, R32tet₃₂ and R48tet₃₂, described above.

20 Example 3C. R96tet₃₂ and R112tet₃₂

Substantially as described in Example 3B, above, R96tet₃₂ and R112tet₃₂ (in which n is 6 and 7, respectively), were expressed in E. coli at approximately the same levels as R48tet₃₂. The starting vector was pR80tet₃₂.

Although some heterogeneity in purified Rtet₃₂ polypeptides was observed by immunoblot analysis, the major reactive species correlated with the band seen by protein staining. The observed molecular weights by SDS-PAGE were approximately twice that expected, although the migration of each of the proteins was proportional to the number of tetrapeptide repeat units in each of the constructs. Amino acid composition determinations on several Rtet₃₂ polypeptides were consistent with expected values.

1 Example 4. R16G

pTerm was prepared by inserting a synthetic linker with the following sequence:

5 5'-GATCCCGGGTGACTGACTGA -3'
3'- GGCCCACTGACTGACTCTAG -5'

into the Bam HI site of pAS1. pAS1 (10 ug) was digested with 25 units of Bam HI. One hundred ng of the Bam HI-cut
10 pAS1 was ligated with 20 nanograms of the synthetic linker and plasmid pTerm was identified with one linker inserted into the Bam HI site of pAS1. This vector retains the Bam HI site and results in the insertion of TGA termination codons downstream of the ATG initiation codon of the cII
15 protein in all three reading frames.

pR16G was prepared by inserting the 192 base pair Xho II fragment from pUC8 clone 1 into the Bam HI site of pTerm and a clone having a single Xho II insert in the proper orientation was selected substantially as described
20 previously.

pR16G was cloned and expressed in E. coli strain N5151, substantially as described above.

R16G has the following sequence:

25 N-met-asp-pro[(asn-ala-asn-pro)₁₅-(asn-val-asp-pro)₁]_n-gly-C
wherein n is one.

30 Since this protein does not contain any aromatic residues it cannot be visualized by Coomassie Brilliant Blue R-250 staining to quantitate expression levels. By immunoblot analysis with 5 monoclonal antibodies specific for the CS protein (Dame et al., cited previously), levels were estimated to be approximately 1% of total cell
35 protein as compared to R16tet₃₂, with which

1 visualization by Coomassie Brilliant Blue R-250 staining
is possible.

Example 4A. R32G, R48G, R64G, R80G and R112G

5 R32G, R48G, R64G, R80G and R112G (R16G in which n
is 2, 3, 4, 5 or 7, respectively) were expressed in E.
coli strain N5151 as described in Example 4, above. These
polypeptides were expressed at about the same level as
R16G. R48G was purified substantially as described in
10 Example 3.

Example 5. R16LA and R32LA

pTerm2 was prepared by inserting a synthetic
linker with the following sequence:

15

5'-GATCCGCTGCGTT -3'
3'- GCGACGCAACTAG-5'

into the Bam HI site of pA51, substantially as described
20 in Example 4. pTerm2 retains the Bam HI site. The 192
base pair Xho II fragment from pUC8 clone 1 was inserted
as described above. pR16LA and pR32LA, clones having one
or two Xho II inserts in the proper orientation,
respectively, were selected substantially as described
25 previously. R32LA was purified substantially as described
in Example 3.

pR16LA and pR32LA were cloned and expressed in E.
coli strain N5151, substantially as described previously.

R16LA and R32LA have the following sequence:

30

N-met-asp-pro[(asn-ala-asn-pro)₁₅(asn-val-asp-pro)₁]_n-
leu-arg-C

wherein n is 1 and 2, respectively. The C-terminal
35 leucine and arginine derive from the synthetic linker in
pTerm2. The R16LA was expressed as about 1% of total E.

- 1 coli protein, whereas, R32LA was expressed at
appr ximately 5% of total cell protein.

Example 6. R16NS1

- 5 pASldeltaEH was prepared by deleting a
non-essential Eco RI - Hind III region of pBR322 origin
from pAS1. Ten micrograms of pAS1 was cut with Eco RI and
Hind III (20 units each) in 200 ul of medium buffer,
treated with DNA polymerase (Klenow), ligated closed, and
10 transformed into E. coli, substantially as described
above. A clone with the 29 base pair Eco RI - Hind III
fragment deleted was identified. A 1236 base pair Bam HI
fragment of pAPR801 (Young et al., Proc. Natl. Acad. Sci.
U.S.A., Volume 80, page 6105 (1983)), containing the
15 influenza virus (A/PR/8/34) NS1 coding region within 861
base pairs of viral origin and 375 base pairs of pBR322
origin, was inserted into the Bam HI site of pASldeltaEH.
The resulting plasmid, pASldeltaEH/801, expresses
authentic NS1 (230 amino acids). This plasmid retains the
20 Bam HI site between the CII translation start site and the
NS1 coding sequence.

- pASldeltaEH/801 (10 ug) was cut with Eco RI (20
units) and Sal I (20 units) in 200 ul of high buffer (50mM
Tris-HCl, pH7.5, 1mM DTT, 10mM MgCl₂, 100mM NaCl) for 2
25 hours at 37°C, treated with DNA polymerase large fragment
(Klenow), and ligated closed, substantially as described
above. A clone having the 650 base pair Eco RI-Sal I
region deleted was isolated. This plasmid, pNSldeltaES,
expresses authentic NS1.

- 30 pR16NS1 was prepared by inserting a 192 base
pair, Xho II fragment from pUC8 clone 1 into the Bam HI
site in pNSldeltaES and clones having a single Xho II
insert in proper orientation were selected substantially
as described previously.

- 35 pR16NS1 was cloned and expressed in E. coli, and
R16NS1 was purified, substantially as described above,
omitting the b iling step.

1 R16NS1 has the following sequence: 0191748

N-met-asp-pr [(asn-ala-asn-pro)₁₅(asn-val-asp-pro)₁]_n-
N227

5 where n is one and N227 is 227 amino acids of NS1 origin.

R16NS1 in the R16NS1 preparation was estimated to
comprise greater than 80% of protein, without the boil or
ion exchange step. R16NS1 represented an especially
10 surprising high proportion, approximately 25%, of total
cellular protein.

Example 6A. R32NS1, R48NS1 and R64NS1

R32NS1 (R16NS1 in which n is 2) was expressed in
15 and purified from E. coli, substantially as described in
Example 3, above, omitting the boiling step. R32NS1 was
expressed at about the same level as R16NS1 and purified
to about the same degree.

R48NS1 (R16NS1 in which n is 3) and R64NS1
20 (R16NS1 in which n is 4) were expressed in E. coli
substantially as described above. R48NS1 and R64NS1
expressed at about 10% and 5% of total E. coli protein,
respectively.

25 Example 7. NS1R48

PR48tet₈₆ was cleaved with Bam HI and
end-filled with DNA polymerase (Klenow) substantially as
described above. The plasmid was then cleaved with Ban II
as described above, to release a 672 base pair fragment
30 carrying 3 Xho II fragments and 96 base pairs from the
tetracycline resistance gene.

Ten micrograms of pAS1deltaEH/801 was cut with
Nco I (20 units) in 200 ul of high buffer for 2 hours at
37°C, and end-filled with DNA polymerase large fragment
35 (Klenow) substantially as described above. The Nco I site
is in the codon for residue 81 in NS1. The plasmid was

1 then cut with Ban II, as described above to delete the
remaining NS1 codons and a portion of the tetracycline
resistance gene, to produce pASIdeltaEH/801-1.

5 The 672 base pair, Bam HI (end-filled)-Ban II
fragment was inserted into pASIdeltaEH/801-1 to prepare
pNS1R48. This plasmid was expressed in E. coli,
substantially as described above. NS1R48 has the
following sequence:

10 N-81N-asp-pro[(asn-ala-asn-pro)₁₅(asn-val-asp-pro)₁]_n
T32-C

wherein 81N is 81 N-terminal amino acids of NS1, n is 3
and T32 is as described above. NS1R48 was expressed as
15 about 5% of total cellular protein.

Example 8. R32N

Ten micrograms of pR32NS1 was cut with Hind III
(25 units) in 200 ul of medium buffer for 2 hours at 37°C,
20 and end-filled with DNA polymerase substantially as
described above, to produce pR32NS1-1. The Hind III site
is in the codon for residue 5 in the NS1 coding region.
pR32NS1-1(100ng) was then ligated closed substantially as
described above. The resulting plasmid, pR32N, now
25 contained a TAA termination codon after the fifth codon in
the NS1 coding sequence. pR32N was used to express R32N
in E. coli substantially as described previously.

R32N has the following sequence:

30 N-met-asp-pro[(asn-ala-asn-pro)₁₅-(asn-val-asp-pro)₁]_n
-N5-C

wherein n is 2 and N5 is 5 amino acids derived from the
NS1 gene. More particularly, N5 has the following
35 sequence:

0191748

1 -asn-thr-val-ser-ser-C.

R32N was expressed as about 5% of total E. coli protein.

5

Example 9. Antibody Response - ELISA

Recombinant proteins R16tet₃₂, R32tet₃₂ and R48tet₃₂ were purified substantially as described above, dialyzed against .01 M phosphate buffered saline, pH 7.0 (PBS), aliquoted, and stored at -80°C. Constructs were mixed with either PBS, aluminum hydroxide (alum) or Complete Freund's Adjuvant (CFA) to yield a 0.5 ml dose containing 50 ug protein. CFA (GIBCO, Grand Island, New York) plus antigen in PBS were emulsified in a 1:1 ratio by agitation for 30 minutes on a mechanical vortexer. Alum was prepared from aluminum hydroxide gel, USP, diluted in PBS. Antigen was absorbed to alum at 4°C for 12 hours on a rotary mixer. The suspension was allowed to settle for an additional 12 hours and sufficient supernatant was discarded to yield 0.80 mg Al and 50 ug recombinant protein per dose. Six to eight week old C57Bl/6 mice were immunized with a total of 50 ug of protein subcutaneously and intraperitoneally (5 animals per group). Animals were boosted 4 weeks after the primary immunization following the same protocol as for the first injections, except that the group which had received the immunogens in CFA were boosted with proteins emulsified in Incomplete Freund's adjuvant (IFA). One week later, whole blood obtained by tail bleeding was pooled, clotted overnight at 4°C, and centrifuged to separate the serum. These sera were stored at -80°C until needed.

An enzyme linked immunosorbent assay (ELISA) was used to test all sera for their ability to react with a 16 amino acid synthetic peptide consisting of four repeats of the P. falciparum CS protein (asn-ala-asn-pro)₄ Dame et

0191748

- 1 al. (Science, Volume 225, page 593 (1984). Synthetic
peptide antigen was coupled to bovine serum albumin (BSA)
was used to coat the wells of microtiter plates. Fifty ul
(0.1 ug) of the screening antigen diluted with 0.01 M
5 phosphate buffered saline, pH 7.4, (PBS), were pipetted
into wells of polystyrene microtitration plates (Immunon
2 Dynatech Laboratories, Alexandria, VA) and held
overnight at room temperature (about 22°C) (RT). Well
contents were then aspirated, filled with blocking buffer
10 (BB= 1.0% BSA, 0.5% casein, 0.005% thimersol and 0.0005%
phenol red in PBS) and held for 1 hour at RT. Mouse sera
were diluted serially in BB and 50 ul was added to each
well. After a 2 hour incubation at RT, wells were
aspirated, washed three times with PBS-0.05% Tween 20
15 (PBS-TW20) and 50 ul of horseradish peroxidase conjugated
to goat anti-mouse IgG (H+L) (Bio-Rad Laboratories,
Richmond, CA) diluted 1/500 with 10% heat inactivated
human serum in PBS was added to each well. After 1 hour,
well contents were aspirated, washed three times with
20 PBS-TW20 and 150 ul of substrate (1 mg 2,2'-azino-di-
(3-ethyl-benzthiazoline sulfonic acid-6) per ml of 0.1 M
citrate-phosphate buffer, pH 4.0, with 0.005% hydrogen
peroxide added immediately before use) was then added to
each well. Absorbance at 414 nm was determined 1 hour
25 later with a ELISA plate reader (Titertek Multiskan, Flow
laboratories, Inc., McLean, VA). The R16tet₃₂,
R32tet₃₂ and R48tet₃₂ constructs all resulted in the
production of antibody which reacted in the
ELISA. R16tet₃₂, when administered alone, was poorly
30 immunogenic when compared to R32tet₃₂ and R48tet₃₂.
Both alum and CFA enhanced immunogenicity of all three
proteins and antibody was detected at titers out to
102,000 in at least one regimen.

35

1 Example 10. Antibody Response - IFA

The antisera from Example 9 were shown to react strongly with authentic P. falciparum CS protein which tested in an indirect immunofluorescent antibody assay (IFA). Reactivity against P. knowlesi, P. cynomologi, P. viva, and P. gallinaceum was not detected. A slight reactivity of the antisera to R32tet₃₂ was seen with P. berghei. This observation is consistent with previous data by Hockmeyer et al., in Proc. 3d Int'l. Symp. Immunobiol. Proteins Peptides, ed. by Atassi, M.Z., Plenum New York (in press) showing that some Mabs to P. falciparum react with P. berghei sporozoites by IFA.

Sporozoites were dissected from the salivary glands of infected mosquitoes substantially as described by Bosworth, J. Parasitol., Volume 61, page 769 (1975), diluted in saline or Medium 199 (GIBCO) containing 0.5% BSA, counted using a haemocytometer and diluted to 2,000-5,000 sporozoites per 10 ul. Ten ul aliquots were spread onto each well of multi-well printed IFA slides, air dried at room temperature and stored at -80°C.

IFA's were initiated by spreading 20 ul volumes of serum, diluted 1/100 with BB, onto the well of an IFA slide containing dried sporozoites. After a 20 minute incubation in a moist chamber at RT, the serum solutions were aspirated and the spots were washed with 2 drops of PBS. Twenty ul aliquots of goat anti-mouse antibody conjugated to fluorescein isothiocyanate (Kirkegard and Perry, Gaithersburg, MD) diluted 1:40 with BB were then added to each spot. After a second 20 minute incubation at RT the spots were again washed with 2 drops of PBS, mounted in glycerol and examined under UV light at 500x magnification for fluorescence.

Example 11. CSP Reaction

Sera from mice immunized with R16tet₃₂, R32tet₃₂ and R48tet₃₂ produced strong CSP positive

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- 1 reactions (Table 1). When administered without adjuvant,
only R16t₃₂ failed to produce antibody which gave
positive CSP reactions, whereas, when given with CFA or
alum, all three constructs induced antibodies, which
5 produced strong CSP reactions.

Table 1. CSP Reactivity of Antisera to R16tet₃₂,
R32tet₃₂, and R48tet₃₂

ADJUVANT	Antisera		
	R16tet ₃₂	R32tet ₃₂	R48tet ₃₂
NONE	0/25(-)	17/25(2+)	21/25(4+)
15 CFA	23/25(4+)	21/25(4+)	21/25(4+)
ALUM	25/25(4+)	25/25(4+)	16/27(2+-4+)

- 20
- CSP reactions were performed essentially as described by Vanderberg et al. Mil. Med. Volume 134 (Supp. 1), page 1183 (1969). Five microliters containing 500-1,000 P. falciparum mosquito salivary gland
- 25 sporozoites resuspended in Medium 199 were mixed with 5 ul of serum on a microscope slide, sealed under a cover slip rimmed with petroleum jelly and incubated at 37°C. for 1 hour. Reactions were evaluated by phase contrast
- microscopy at 400X magnification. Twenty-five random
- 30 sporozoites were examined for each serum sample and the number of CSP positive organisms are indicated. The degree of CSP reactivity as described by Vanderberg et al., cited above, is shown in parentheses. A (-) indicates no CSP reactivity detectable; (2+) indicates
- 35 appearance of a granular precipitate on the surface of the sporozoites; (4+) indicates appearance of a long,

1 thread-like filament at one end of the sporozoites.
Normal mouse serum, and serum from mice immunized with CFA
alone, produced no detectable CSP reactivity in parallel
assays.

5

Example 12. Hepatocyte Blocking

The sera from Example 9, above, were examined in
an in vitro inhibition of invasion assay (Table 2). These
data show that the R32tet₃₂ and R48tet₃₂ proteins
10 induce antibodies with strong blocking activity even in
the absence of adjuvant. R16tet₃₂ was less efficient in
eliciting strong blocking antibodies except when
administered adsorbed to alum. This finding is consistent
with the poor CSP reactivity and low ELISA titers observed
15 with the antisera raised to the R16tet₃₂ protein.

Table 2. Inhibition of P. falciparum Sporozoite Invasion
HepG2 - A16 Hepatoma cells in vitro.

20

<u>ADJUVANT</u>	<u>Antisera</u>		
	<u>R16</u>	<u>R32</u>	<u>R48</u>
NONE	46	95	92
CFA	76	92	94
25 ALUM	100	100	96

Inhibition of sporozoite invasion of cultured
cells was performed substantially as previously described
30 by Hollingdale et al. J. Immunol. Volume 32, page 909
(1984). The sera obtained from mice immunized with the
R16tet₃₂, R32tet₃₂ and R48tet₃₂ constructs were
tested for their ability to inhibit invasion of cultured
cells by P. falciparum sporozoites. The sera were diluted
35 in culture medium and added to HepG2-A16 cell cultures to
yield a final dilution of 1:20 (V/V). Cultures then

0191748

1 received 12,000 to 40,000 mosquit salivary gland P.
falciparum sporozoites and were incubated at 37°C in 5%
CO₂ atmosphere for 3 hours, rinsed with Dulbecco's
phosphate-buffered saline (PBS), fixed in methanol, and
5 rinsed 2 times with PBS.

Sporozoites that had entered cells were
visualized by an immunoperoxidase antibody assay (IPA)
(Hollingdale et al., cited above). The IPA was carried
out by first treating the fixed cultures with a Mab to
10 P. falciparum (2F1.1, See, Dame et al., cited above)
followed by incubation with rabbit anti-mouse
immunoglobulin conjugated with horseradish peroxidase and
staining with 3,3-diaminobenzidine. The number of
sporozoites that invaded cultured cells was determined by
15 counting the intracellular parasites present in the entire
preparation on a Leitz microscope at 250X with a dark blue
filter. Experiments were carried out either in duplicate
or triplicate and each cell culture within an experiment
received an equal number of sporozoites. Inhibition was
20 the percentage reduction of sporozoite invasion by
anti-constrict immune sera compared to normal mouse serum
controls where CS reactive Mab 2F1.1 gave 100% inhibition
of sporozoite invasion at dilutions of 1/20.

Recombinant proteins RLA, R16NS1 and R32NS1,
25 prepared substantially as described above, were similarly
tested by the ELISA and IFA assays and were shown
similarly to induce antibody which reacted with the 16
residue synthetic peptide and to give positive CSP
reactions. R32tet₃₂ and R32LA are preferred, because of
30 their relative homogeneity, expression levels, and ease of
preparation.

Of primary interest in any synthetically produced
vaccine, is whether antibody produced against the
synthetic immunogen will recognize the authentic molecule
35 and whether the antibody will possess the necessary

0191748

1 biological properties to confer protection. The Examples
showing both an immunofluorescence assay and the CSP
reaction demonstrate that antibody produced against the E.
5 coli constructs reacts with the surface of the sporozoite
and thus recognizes authentic CS protein. The presence of
CSP antibody has been shown in animals and man to be an
important correlate of protective immunity. The fact that
anti-construct antibodies inhibit sporozoite invasion of
human hepatoma cells in vitro is significant. Hollingdale
10 et al., cited above, showed that both Mabs against P.
falciparum and P. vivax as well as polyclonal serum from
humans immune to these malaria species blocked sporozoite
invasion. Blocking of sporozoite invasion in vitro is
thus considered to be an assay for protective antibody.
15 Thus, the data collectively demonstrates that the vaccine
of the invasion can be used to protect humans from
infection by P. falciparum sporozoites.

The immune response to these recombinant proteins
as assessed by ELISA titer, surface reactivity (as shown
20 by IFA and CSP) and blocking of sporozoite invasion is
enhanced by use of either Complete Freund's Adjuvant or
Alum. Complete Freund's Adjuvant can not be used in humans
since it causes fever, produces granulomas and results in
tuberculin hypersensitivity. Alum is currently used as an
25 adjuvant in established vaccines such as diphtheria and
tetanus toxoid as well as one of the newest vaccines,
Hepatitis B. It has proven efficacy and a long history of
safe use in man.

30 Example 13. Vaccine Preparation

An illustrative vaccine is prepared as follows.
To a buffered, aqueous solution of 3% aluminum hydroxide
(10 mM sodium phosphate, 150 mM NaCl, pH 6.8; sterilized
by filtration), the polypeptide of the invention in
35 similar buffer is added with stirring to a final
concentration of 100 ug/ml of polypeptide and 1.0 mg/ml of

0191748

- 1 aluminum (Al^{3+}). Th pH is maintained at 6.6. The
mixture is left overnight at about 0°C. Thimersol is
added to a final concentration of 0.005%. The pH is
checked and adjusted, if necessary, to 6.8.
- 5 While the above fully describes the invention and
all preferred embodiments thereof, it is to be appreciated
that the invention is not limited to the embodiments
particularly described but rather includes all
modifications thereof coming within the scope of the
10 following claims.

15

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CLAIMS for the Contracting States :

1 BE - CH - DE - FR - GB - IT - LI - LU - NL - SE

1. An E. coli expression vector having a coding sequence for all or a portion of the repeat unit of the CS protein of Plasmodium falciparum operatively linked to a
5 regulatory element.

2. The vector of claim 1 which comprises the Xho II - Xho II region of the CS protein coding sequence or tandem repeats thereof.

3. The vector of claim 1 in which the coding
10 sequence codes for at least 4 tandem repeats.

4. The vector of claim 3 in which the coding sequence codes for about 16 to 148 repeat units.

5. The vector of claim 4 in which the coding sequence codes for a polypeptide selected from the group
15 consisting of Rtet₃₂ polypeptides, Rtet₈₆ polypeptides, RNS1 polypeptides, NS1R polypeptides, RG polypeptides, RLA polypeptides, and RN polypeptides.

6. The vector of claim 4 in which the coding sequence codes for a polypeptide selected from

20	R16tet ₈₆	R32G
	R32tet ₈₆	R48G
	R48tet ₈₆	R64G
	R16tet ₃₂	R80G
	R32tet ₃₂	R112G
25	R48tet ₃₂	R16LA
	R64tet ₃₂	R32LA
	R80tet ₃₂	R16NS1
	R96tet ₃₂	R32NS1
	R112tet ₃₂	R48NS1
30	R16G	R64NS1
	NS1R48	R32N.

7. The vector of claim 4 in which the polypeptide is R32tet₃₂ or R32LA.

8. The vector of claim 1 in which the regulatory
35 element comprises the PL promoter and the cII ribosome binding site including the cII translation initiation site.

- 1 9. The vector of claim 8 having the pAS1
regulatory elements.
10. An E. coli transformed with the vector of
claim 1.
- 5 11. An E. coli transformed with the vector of
claim 2.
12. An E. coli transformed with the vector of
claim 3.
13. An E. coli transformed with the vector of
10 claim 4.
14. An E. coli transformed with the vector of
claim 5.
15. An E. coli transformed with the vector of
claim 6.
- 15 16. An E. coli transformed with the vector of
claim 7.
17. An E. coli transformed with the vector of
claim 8.
18. An E. coli transformed with the vector of
20 claim 9.
19. A method of purifying a polypeptide having
four or more tandem repeat units of the Plasmodium
falciparum CS protein from a clarified cell extract of
producing E. coli which comprises addition of a detergent
25 to the cell extract followed by heating of the extract to
precipitate bacterial proteins and then further purifying
the polypeptide from the supernatant.
20. A method of producing a polypeptide having
for or more tandem repeat units of the Plasmodium
30 falciparum CS protein comprising culturing the E. coli of
claim 10 such that the polypeptide is produced and
purifying the polypeptide therefrom.

35

CLAIMS for the Contracting Stat AT

1. A process for preparing a polypeptide which
 can be used in a vaccine to protect humans against
 5 infection by Plasmodium falciparum comprising inserting
 a coding sequence for all or a portion of the repeat
 unit of the CS protein of Plasmodium falciparum into an
E. coli expression vector such that the coding sequence
 is operatively linked to a regulatory element, cultu-
 10 ring the transformed E. coli such that the polypeptide
 is produced and purifying the polypeptide therefrom.

2. The process of claim 1 wherein the coding
 sequence for the repeat unit or for the portion of the
 15 repeat unit is the XhoII-XhoII region of the CS protein
 coding sequence or tandem repeats thereof.

3. The process of claim 1 which comprises inser-
 ting a coding sequence for at least 4 tandem repeat
 20 units into the expression vector.

4. The process of claim 3 which comprises inser-
 ting a coding sequence for 16 to 48 tandem repeat units.

25 5. The process of claim 4 in which the coding
 sequence codes for a polypeptide selected from the
 group consisting of Rtet₃₂ polypeptides, Rtet₈₆
 polypeptides, RNS1 polypeptides, NS1R polypeptides, RG
 polypeptides, RLA polypeptides, and RN polypeptides.

30 6. The process of claim 4 in which the coding
 sequence codes for a polypeptide selected from

	R16tet ₈₆	R32G
	R32tet ₈₆	R48G
35	R48tet ₈₆	R64G

	R16tet ₃₂	R80G
	R32tet ₃₂	R112G
	R48tet ₃₂	R16LA
	R64tet ₃₂	R32LA
5	R80tet ₃₂	R16NNS1
	R96tet ₃₂	R32NS1
	R112tet ₃₂	R48NS1
	R16G	R64NS1

10 7. The process of claim 4 in which the polypeptide is R32tet₃₂ or R32LA.

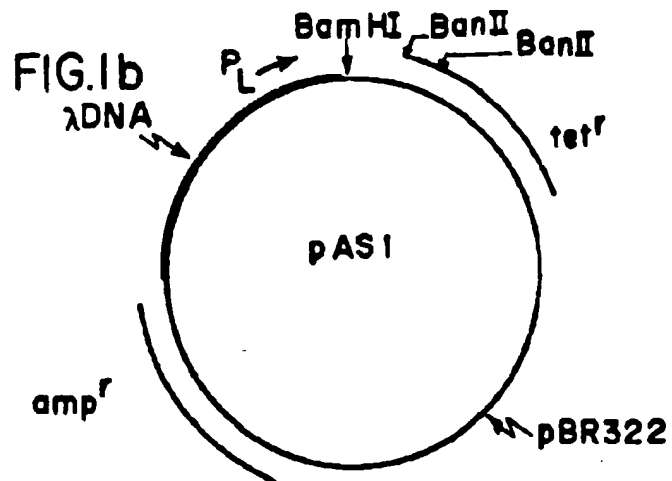
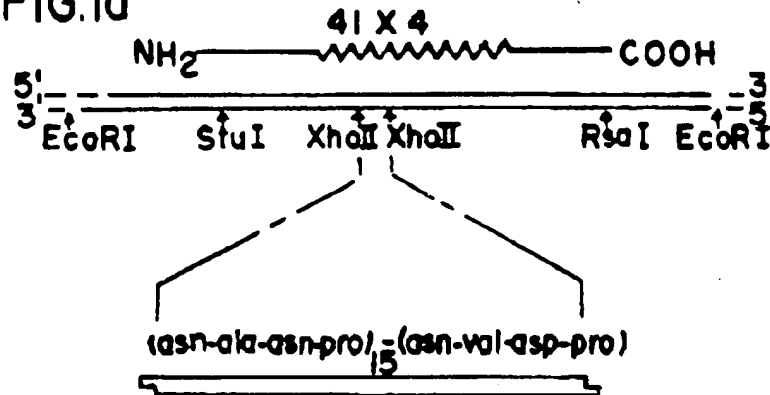
8. The process of claim 1 in which the regulatory element comprises the PL promoter and the cII ribosome
15 binding site including the cII translation initiation site.

9. The process of claim 8 in which the regulatory element is the regulatory element of pAS-1.

20

10. The process of claim 1 in which the polypeptide is purified by adding a detergent to a cell extract of the E. coli transformants, heating the extract to precipitate bacterial proteins and then fur-
25 ther purifying the polypeptide from the supernatant by standard protein purification techniques.

FIG. 1a





DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
Y,D	SCIENCE, vol. 225, 10th August 1984, pages 593-599; J.B. DAME et al.: "Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite Plasmodium falciparum" * Figure 3 *	1-20	C 12 N 15/00 A 61 K 39/015
Y,D	--- METHODS IN ENZYMOLOGY, vol. 101, part C, 1983, pages 123-138, Academic Press Inc.; M. ROSENBERG et al.: "The use of pKC30 and its derivatives for controlled expression of genes" * Figures 4,6 *	1-20	
X,P	--- SCIENCE, vol. 228, 24th May 1985, pages 958-962; J.F. YOUNG et al.: "Expression of Plasmodium falciparum circumsporozoite proteins in Escherichia coli for potential use in a human malaria vaccine" * Whole article *	1-20	TECHNICAL FIELDS SEARCHED (Int. Cl. 4) C 12 N A 61 K C 12 P
Y,P	--- EP-A-0 166 410 (THE USA) * Figure 2 *	1-20	
Y,P	--- EP-A-0 153 188 (NATIONAL RESEARCH DEVELOPMENT CORP.) * Figure 4 *	1-20	
--- -/-			
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14-05-1986	Examiner CUPIDO M.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p>			

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DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
T	BIOTECHNOLOGY, vol. 3, no. 8, August 1985, pages 729-740, New York, US; J.V. RAVETCH et al.: "Molecular genetic strategies for the development of anti-malarial vaccines" * Page 733 * -----	1-20	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 14-05-1986	Examiner CUPIDO M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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